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Protein-free media for Leptospira canicola.

by H. Woratz.

Translated from Zentralbl. f. Bakt. I Abt. Orig. 162:106-111 (1955) by the Technical Library, Technical Information Division.

Ungermann's (12) recipe for artificial culture of pathogenic leptospirae in rabbit serum diluted with tap water has been used in laboratories with few modifications; the latter involved only buffer capacity or addition of peptone.

Extensive studies aimed at the replacement of rabbit serum by more accessible substrates were initiated. Donkey, horse, sheep and guinea pig sera have been utilized with varying degrees of success, without, however, becoming permanently established.

Brede and coworkers (1) grew cultures on the allantoic fluid of chick embryos. Extracts of E. coli cultures mixed with sterile garden soil were described by Kiktenko (9) and Hein (8). High yields were obtained by Gram and Schlipkoeter (2) and Gram (3) with "T-vitamin Goetsch," yeast and liver extracts. The task of discovering the essential growth-promoting factors was undertaken with particular thoroughness by M. R. Greene and coworkers (4,5,6,7): According to their results, a large number of amino acids and growth substances are unimportant for leptospirae. They grew many types of pathogenic leptospirae in 14 passages through a nutrient medium containing a few salts, thiamine, asparagin and electrophoretically pure rabbit serum albumin. Since the authors were unable to replace the rabbit serum albumin, they concluded that rabbit serum albumin possesses a specific property per se. They did not determine whether the essential factor consists of a large protein molecule or merely of fractions.

Marshall (10) thinks that rabbit serum can fix toxic substances.

If Marshall's assumption is correct, there must be other substances capable of absorbing toxic products, as in the case of serum. The results of Zironi (13). who recultivated the same medium six times after removing dense cultures by centrifugation, speak against the accumulation of toxic products of metabolism. Serum albumin apparently constitutes the nutrient substrate for leptospirae.

Judging by current experience with synthetic media used in connection with other bacterial species, it is improbable that whole protein molecules are taken up by leptospirae; most likely small protein fractions or wholly unrelated substances are important for their metabolism. Since studies of leptospiral nutrients lose their perspicuity upon addition of native serum, such investigations of growth-promoting factors encounter considerable difficulty. The task would be simplified by the discovery of a basic material that contains copious amounts of these substances. Our studies were designed to test a number of other substances for their content of growth-promoting factors, coupled with subsequent attempts to enrich the substances in question.

Extensive pilot tests extending over several years due to the slow growth rate of leptospirae had examined the following substances for their effect, some in several charges: Hen's egg yolk and albumin, cow's milk and aqueous extracts from liver and kidney, alcoholic extracts of bovine heart, fractions of rabbit, sheep, cow and horse serum prepared with ammonium sulfate, uranium acetate or trichloroacetic acid. The media of Brede et al. (1), Kiktenko and Hein (9,8), Greene et al. (6) were also included in the scope of this study.

Growth of leptospirae was observed in all media containing protein serum fractions and in egg yolk. The action of cow's milk, liver and kidney extracts was questionable. No favorable effect was seen in egg albumin and in serum fractions from which protein had been precipitated with ammonium sulfate or trichloroacetic acid. Preparations of media after Brede et al. (1), Kiktenko (9) and Hein (8) led to variable results, especially in later passages, as could be expected in view of the great and well-known difficulties that beset work with leptospirae. The nutrient proposed by Greene and coworkers (6) -- containing rabbit serum albumin, thiamine chloride and asparagin as essential components -- has given good results.

These orienting studies disclosed that the growth of leptospirae (principally Leptospira canicola) is promoted not only by addition of egg yolk and serum fractions, but also by alcoholic extracts of bovine heart. Since the latter did manifest a certain increase in the desired substances, it seemed justified to search for the growth-promoting factors in other alcoholic extracts, e.g., in rabbit serum, egg yolk and commercial citochol extract.

In order to make such tests possible, the leptospirae were grown in a protein-free basic solution in which they remained motile for extended periods. Leptospirae remained viable for up to 2 weeks without showing marked reproduction in a solution containing Korthof's peptone-salt mixture as well as asparagin and thiamine hydrochloride (Greene 7). Alcoholic extracts were added to this basic mixture. The following test procedure was adhered to:

Test procedure

I. Basic mixture.

All glassware was used only after repeated rinsing with redistilled water.

The basic mixture contains the following substances:

a)	Casein peptone	400 mg	$CaCl_2$		40 mg
	NaCl	1400 mg	KH_2PO_4		180 mg
	$NaHCO_3$	20 mg	Na_2HPO_4		960 mg
	KCl	40 mg	distilled water	to	200 ml

These substances were dissolved in the autoclave, filtered through filter paper after cooling, distributed on tubes in lots of 10 ml and sterilized. Basic mixture a) is Korthof's salt-peptone solution concentrated 5 times; this form simplifies laboratory procedure.

b) Greene's solution must be freshly prepared before use:

asparagin	200 mg	dissolved in dist. water
thiamine hydrochloride	0.2 mg	

II. Preparation of ethanol extracts.

a) Rabbit serum.

36 ml of rabbit serum were frozen at -20°C in an Erlenmeyer flask with ground stopper and covered with a 5-fold amount of 96% ethanol cooled to -20°C. Repeated shaking caused the frozen serum to be dissolved and slowly extracted in the next 3 days. Precipitated serum protein was removed by filtration. The clear filtrate was placed in dialyzing tubes; the alcohol was evaporated overnight (with the exception of a small residue) in the incubator at 37°C. The dialyzing tube was then filled with 100 ml of redistilled water, thoroughly kneaded and dialyzed for 6 days against redistilled water (changed daily). The contents of the dialyzing tube were expanded to 200 ml with distilled water, 40 ml of the peptone-salt solution, 200 mg asparagin and 0.2 mg thiamine. 14 x 140 mm tubes were charged with lots of 5 ml and heated for 15 minutes in the autoclave.

b) Egg yolk extract.

1 egg yolk was vigorously shaken with glass beads in a 500 ml ground flask, frozen at -20°C and mixed with 60 ml of ethanol at the same temperature. Extraction for 6 days at -20°C with repeated, vigorous shaking. After centrifugation at 1200 g for 15 minutes the

clear, yellowish supernatant was dialyzed for 6 days against redistilled water. Dialysis produced 135 mg, which were expanded to 200 ml with 40 ml of basic mixture a), 200 mg asparagin, 0.2 mg thiamine chloride and redistilled water. Decanting into tubes and heating yielded a white, turbid liquid. Preliminary tests proved this solution to be suitable only in dilution with basic mixture a) and b) at a ratio of 1:40, since leptospirae were killed by stronger concentrations.

c) Extract of bovine heart.

10 ml of commercial bovine heart extract for the WaR were dialyzed in the dialyzing tube for 6 days against redistilled water, which was changed every 24 hours. This was followed by centrifugation for 1 hour at 1200 g. The surface membrane (= lesterin) was discarded, the supernatant was filled to 200 ml with the appropriate amount of basic mixtures a) and b) and redistilled water. Other steps as above.

d) Citochol extract.

10 ml of commercial extract for the citochol reaction were prepared in the same manner as bovine heart extract.

III. Culture of leptospirae.

All tests employed leptospiral strains Canicola Utrecht and Zurich which, in contrast to Weil and harvest fever strains, proved to be resistant. As is customary in work with leptospirae, the inoculum was 1/20 the amount of nutrient solution (= 0.25 ml). Microscopic evaluation of cultures and initiation of new passages were carried out at intervals of 8-10 days.

Since serum fractions in the cultures of institutional collections were to be excluded, results were evaluated only after leptospirae had been grown in several passages through the new milieu. Washing of leptospirae was dispensed with due to the danger of contamination.

Results

After orienting tests had disclosed growth-promoting substances in alcoholic extracts of bovine heart, egg yolk and serum, we initiated continuous passages through this nutrient milieu with Leptospira canicola Utrecht and L. can. Zurich. We succeeded in carrying strain Utrecht to the 11th passage in bovine heart media, after which the culture was contaminated by staphylococci. A tube of the 5th passage found by chance 2 months later permitted the continuation of subcultures. After 7 additional passages (for a total of 13) the culture was again ruined by staphylococci; the series was discontinued and restarted from a serum water culture.

While strain Utrecht was grown principally in bovine heart solution, and the performance of egg yolk and serum extracts was merely tested in parallel studies, strain Zurich was continued from the 6th passage in several parallel series of different media. Strict care was taken that leptospirae were transferred to the same type of nutrient whenever a new passage was initiated. New passages were started from cultures 8-10 days old. The microscopic evaluation of the 6th, 7th and 8th passages is tabulated as an example.

Strain L. can. Zurich now exists in its 25th passage. As evident from the table, the leptospirae grow quite well in these media, but never reach the growth density of serum water cultures. With the exception of serum extract preparations, these media are optically objectionable, since disinfection in the autoclave was chosen instead of sterile filtration through Seitz filters with its inherent inconveniences (shifts in pH, Ca and Fe ions). All attempts to cultivate harvest fever and Weil strains in these solutions have been unsatisfactory.

Discussion

The preparation of ethanol extracts from rabbit serum, egg yolk and bovine heart excludes proteins as essential growth-promoting factors for Leptospira canicola. Since solutions deproteinated in precipitation tests with trichloroacetic acid and ammonium sulfate are ineffectual, in contrast to protein fractions, it is possible that the unknown substances settle out with the protein. The weak activity of heated media presumably is based on a similar effect. During treatment with ethanol, the growth-promoting substances at least go into partial solution. They are thermostable at 100°C and 15 minutes activity. Longer durations and higher temperatures were not tested.

Although the substances in question are difficult to dialyze, as indicated by a duration of 6 days, no data on their dialyzability may be gained therefrom.

As evident from dilutions made during preparation of egg yolk extract, leptospirae become motionless after a few hours in a concentrated solution. Reproduction appears only in greater dilutions. It is not clear whether the unknown substances are inhibitory in greater amounts or whether entirely different substances, whose activity ceases upon dilution, have this effect.

Since Leptospira icterogenes and L. grippotyphosa could not be cultivated for long in these media, these substrates may possibly serve as differential media, but this problem must be clarified in thorough studies.

Table 1

		Serum extract	Egg yolk extract	Bovine heart extract	Citochol extract
6th passage:					
8 days after initiation	1.	4	4	3	Ø
	2.	4	5	2	2
	3.	4	5	2	2
16 days after initiation	1.	4	4	3	3
	2.	4	4	3	5
	3.	4	4	4	5
24 days after initiation	1.	2	3	5	V
	2.	1	2	4	5
	3.	2	3	5	5
7th passage:					
8 days after initiation	1.	4	4	4	4
	2.	4	4	4	4
	3.	Ø	5	4	4
16 days after initiation	1.	4	4	5	5
	2.	2	1	5	5
	3.	Ø	2	5	5
24 days after initiation	1.	2	3	4	5
	2.	1	1	5	5
	3.	Ø	2	5	5
8th passage:					
8 days after initiation	1.	4	1	1	2
	2.	4	2	1	2
	3.	4	1	2	2
16 days after initiation	1.	Ø	5	5	5
	2.	4	5	4	5
	3.	4	5	5	5
24 days after initiation	1.	Ø	5	5	5
	2.	4	5	5	5
	3.	4	5	5	5

The table shows the growth of L. can. Zurich in media with alcoholic extracts. Intensity of growth is listed according to Rimpau's (11) system: at magnification 270, Ø = 0; 1 = 1-10; 2 = 20-30; 3 = 30-50; 4 = 50-100; 5 = countless leptospirae in the field; V = contaminated by other bacteria. Passages were always continued on the same type of medium. Three new tubes were inoculated with material from the most densely overgrown culture.